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Effects of pertussis toxin on the behavioural and ECoG spectrum changes induced by clonidine and yohimbine after their microinfusion into the locus coeruleus

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1 Pertussis toxin, a substance which interferes selectively with receptor-mediated signal transduction mechanisms, was injected into the locus coeruleus of rats 1, 2, 3, 6 or 10 days before the microinjection of clonidine or yohimbine into the same site.

2 Clonidine produced in control rats typical behavioural sedation and/or sleep and ECoG synchronization while yohimbine produced behavioural arousal and ECoG desynchronization.

3 The behavioural and ECoG effects of both compounds were blocked in animals pretreated with pertussis toxin. This activity was more marked from 2 to 6 days after pertussis toxin pretreatment and was restored 10 days after toxin administration. In addition, the behavioural and ECoG slow-wave sleep observed after intraperitoneal administration of clonidine ($0.2\,\mu mol\,kg^{-1}$) was significantly reduced by prior (3 days) microinfusion of pertussis toxin into the locus coeruleus.

4 These results are consistent with the hypothesis that the behavioural and ECoG effects of clonidine and yohimbine are mediated via a guanine regulatory protein which is affected by pertussis toxin.

Introduction

It is widely accepted that the molecular events subsequent to α_2-adrenoceptor stimulation involve the inhibition of adenylate cyclase via the inhibitory guanosine 5'-triphosphate (GTP) binding regulatory protein (Gi) in a variety of tissues and cells (see Aghajanian & Wang, 1986). Recently the availability of agents such as cholera and pertussis toxin (PTx), which interfere selectively with receptor-mediated signal transduction processes (Bokoch *et al.*, 1983; Abood *et al.*, 1985; Ohta *et al.*, 1985), has been used as a valid tool to improve our knowledge of cellular events leading to the pharmacological action of compounds acting on α_2-adrenoceptors. In particular, PTx has been shown to prevent receptor-mediated adenylate cyclase inhibition by adenosine 5'-diphosphate (ADP) ribosylation of a nucleotide binding protein (Gi) in the cell membrane (Hazeki & Ui, 1981; Jakobs *et al.*, 1984).

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In previous experiments we have shown that clonidine microinfused directly into the rat locus coeruleus (LC) at extremely low doses produces dose-dependent behavioural sedation and/or sleep accompanied by a significant increase in total voltage power with a predominant increase in the lower frequency bands (De Sarro *et al.*, 1987).

In the present study we planned to ascertain whether the intracerebral injection of PTx was able to affect the behavioural and electrocortical spectrum power changes induced by selective α_2-adrenoceptor agonists, i.e. clonidine or antagonists, i.e. yohimbine, after their infusion into the locus coeruleus of the rat. This could give some indication as to whether the α_2-adrenoceptors located on the noradrenergic neurones in the locus coeruleus are linked in a negative manner to adenylate cyclase.

Methods

Male Sprague Dawley rats (50–70 days old; 200 ± 20.2 g) were purchased from Charles River,

(Calco, Como, Italy) and housed in stable conditions of humidity (60 ± 5%) and temperature (22 ± 2°C). They were fed with standard diet and had water *ad libitum*. Animals were maintained on a 12 h light and 12 h dark cycle (lights on 06 h 00 min–18 h 00 min, off 18 h 00 min–06 h 00 min).

Rats were stereotaxically implanted under choral hydrate anaesthesia (400 mg kg^{-1} i.p.; Carlo Erba, Milan) with stainless steel guide cannula (25 gauge), according to the atlas coordinates of Paxinos & Watson (1982), to permit drug microinfusion into the locus coeruleus.

After surgery a minimum of 24 h was allowed before experiments were carried out. All experiments were performed beginning at approximately 10 h 00 min. Freely moving rats were microinjected via an injector cannula (28 gauge) which extended to approximately 2 mm below the tip of the guide cannula.

Electrocortical (ECoG) activity was recorded via 4 chronically and symmetrically implanted steel screw electrodes inserted onto each fronto-parietal cortex (±2 mm behind the bregma and ±2 mm lateral to the midline) by a Stoelting stereotaxic frame. The ground electrode was implanted epidurally over the nasal bone. All electrodes and the injection guide cannula were anchored to the skull by acrylic dental cement. Electrocortical activity was recorded by means of an 8 channel EEG machine (OTE BIOMEDICA, Florence). For statistical purposes, the quantification of total voltage power (0–16 Hz) and of single frequency bands (0–3; 3–6; 6–12; 12–16 Hz) was carried out by using a Berg-Fourier analyser (OTE BIOMEDICA) according to De Sarro *et al.* (1987). ECoG power spectra were obtained by averaging spectra derived from 5 min ECoG epochs and the integrated energy signals were expressed as $\mu V^2 s^{-1}$; the time constant (0.03 s) was short enough to reduce the number of artefacts (HF cut-off = 5.3 Hz).

The behavioural and electrocortical spectrum power effects were studied in rats randomly divided into two groups. The first group was treated with PTx, while the second group received the vehicle in which PTx was dissolved (67 mM sodium phosphate buffer pH 7.4). The experiments with clonidine and yohimbine were carried out 1, 2, 3, 6 and 10 days after the administration of PTx or vehicle.

In addition, another 12 rats were randomly separated into two groups, the first group receiving PTx, the second treated with vehicle. In this case clonidine was administered intraperitoneally 3 days after the administration of PTx or vehicle.

The animals, placed individually in transparent cages (35 × 35 × 25 cm), were allowed 60 min before drug administration to acclimatize to the new environment. The behavioural changes and their onset and duration were recorded after drug injection. Each animal was used only once.

Post-mortem histological examination confirmed the location of the guide cannula. Only animals in which the location of the injection site was confirmed histologically were used in the analysis of behavioural and ECoG data.

To quantify changes of total voltage power and of preselected bands of frequency induced by clonidine or yohimbine in rats pretreated with PTx or vehicle, the area (expressed in mm^2) under the curve corresponding to plotted total voltage values during 30 min periods after each compound was integrated by means of a Commodore computer and the percentage changes of the integrated area in comparison to the same interval area during the pretreatment period were calculated according to the 'trapezoidal rule' (Tallarida & Murray, 1981). To reduce inter-animal variation of baseline electrocortical activity and of single frequency bands, the percentage changes following drug treatment were compared to the values of the corresponding period before treatment by use of analysis of variance (ANOVA) followed, where appropriate, by analysis with Student's *t* test. The number of experiments are shown in parentheses.

Drugs

Purified pertussis toxin (gift from Dr E. Relyveld, Institut Pasteur, Garches, France) or vehicle were injected into the locus coeruleus in a volume of a 0.5 µl the day of stereotaxic implantation of cannula and electrodes.

Clonidine hydrochloride (Boehringer-Ingelheim, Germany) and yohimbine hydrochloride (Sigma St. Louis, MO, U.S.A.) were infused into the locus coeruleus by using a 5 µl Hamilton syringe, connected by a teflon tube to an injector cannula. The drug was infused in a volume of 0.5 µl at a rate of 0.2 µl min^{-1} and the cannula left *in situ* for a further 1 min.

Results

Clonidine and yohimbine effects in control rats

The behavioural and ECoG spectrum power changes following focal microinfusion of clonidine (0.56 and 1.2 nmol) or yohimbine (1.3 and 2.6 nmol) into the LC were similar to those already obtained (De Sarro *et al.*, 1987). In particular, clonidine induced typical sedation and/or sleep and ECoG synchronization with a periodic increase in total voltage power, predominantly in the 0–3, 3–6 and 9–12 Hz frequency bands, lasting from 125 to

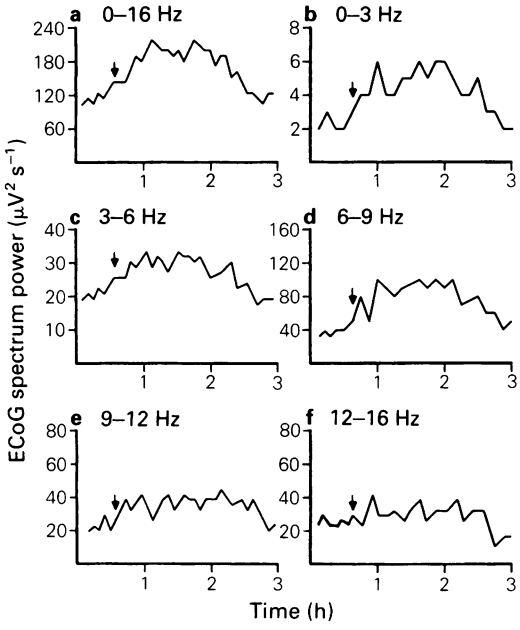

Figure 1 Effects of a single microinjection into the locus coeruleus of clonidine (0.56 nmol at arrow) on electrocortical spectrum power. Note the significant increase in (a) total and (b) 0–3, (c) 3–6, (d) 6–9 and (e) 9–12 Hz voltage power. The slight increase in (f) 12–16 Hz band was not statistically significant.

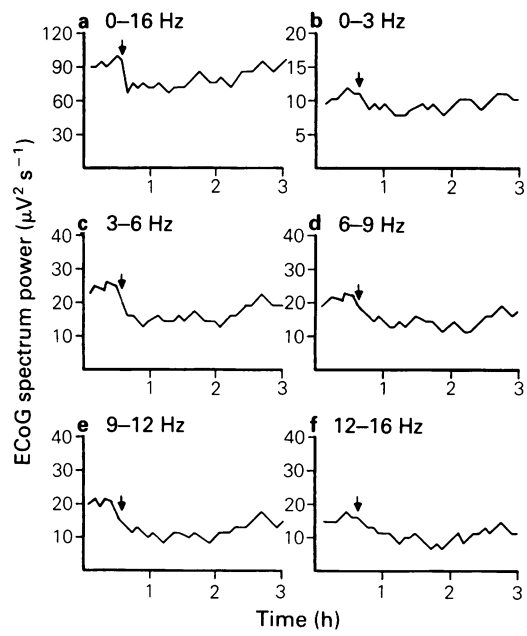

Figure 2 Effects of a single microinjection into the locus coeruleus of yohimbine (2.6 nmol at arrow) on electrocortical spectrum power. Note the significant fall in (a) total and in (c) 3–6, (d) 6–9 and (e) 9–12 Hz voltage power.

220 min according to the dose. In contrast, yohimbine produced behavioural arousal, an increase in locomotor and exploratory activity and ECoG desynchronization, which were associated with a significant decrease in total voltage power and that in the 3–6, 6–9 and sometimes in the 9–12 Hz frequency bands lasting from 60 to 160 min depending on the dose. Figures 1 and 2 show some typical examples of the ECoG changes which usually occur following clonidine and yohimbine infusion into the LC.

Clonidine and yohimbine effects in PTx pretreated rats

Preliminary experiments have indicated that PTx (0.34, 0.68 and 3.4 μg) was only minimally effective within the day of injection, while at 1, 2, 3, 6 days after the administration of PTx (0.34, 0.68 and 3.4 μg) the rats ($n = 6$ for each group) usually displayed 'hyperalgesia' (i.e. they squealed when handled or touched) and showed an alert state after sound or touch stimuli. However, no significant changes in the ECoG spectrum were observed.

Pretreatment with PTx (0.34 μg in 0.5 μl) produced a gradual decrease of the responsiveness of the LC neurones to clonidine (Figure 3) and yohimbine (Figure 4).

No significant changes of the effects of clonidine (0.56 and 1.2 nmol) and yohimbine (1.3 and 2.6 nmol) were observed, in comparison to vehicle-treated animals, 1 day after PTx treatment. In contrast, 2, 3 and 6 days after toxin treatment the behavioural and ECoG spectrum power effects induced by clonidine and yohimbine were reduced, abolished or reversed in a dose- and time-dependent manner (Tables 1 and 2; Figures 3 and 4). In particular, a tendency to reduce significantly or modify the responsiveness of the LC neurones to the action of clonidine and yohimbine was observed 2 days after PTx and it was more markedly reduced or almost absent 6 days after the pretreatment with PTx. However, the clonidine ECoG synchronizing response and yohimbine desynchronizing effect were restored 10 days after PTx treatment ($n = 8$ animals).

Intraperitoneal administration of clonidine (0.2 μmol kg^{-1}) also induced, in rats injected into the locus coeruleus with vehicle, behavioural and electrocortical soporific effects with an increase in total voltage power and that in the 0–3, 3–6, 6–9 and 9–12 Hz frequency bands, whereas rats pretreated (3 days before) with PTx showed a slight and short

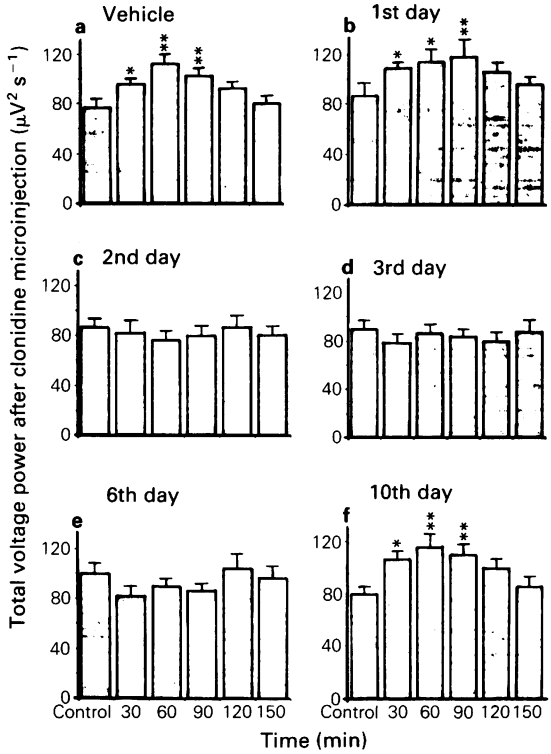

Figure 3 Time course of the effects of an infusion into the locus coeruleus of clonidine (0.56 nmol in 0.5 μl) on the ECoG total voltage power at various times after pretreatment, into the same brain site, with pertussis toxin (PTx) (0.34 μg in 0.5 μl). Each column represents the mean (bars show s.e.mean) of at least 6 observations. Groups of (a) vehicle- and (b–f) PTx-treated rats were previously analysed by use of ANOVA followed, where appropriate, by paired Student's *t* test. Significance of the differences of total voltage power between pretreatment period and post-drug periods are denoted: * $P < 0.05$ and ** $P < 0.01$.

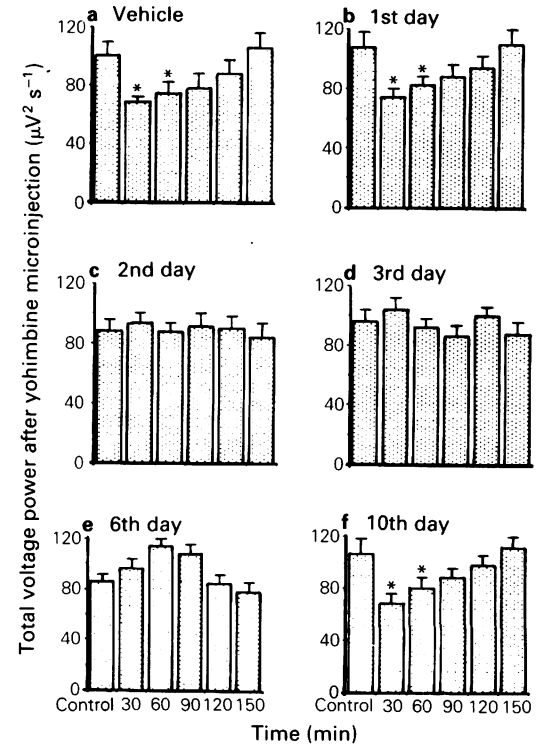

Figure 4 Time course of the effects of an infusion into the locus coeruleus of yohimbine (2.6 nmol in 0.5 μl) on the ECoG total voltage power at various times after pretreatment, into the same brain site, with pertussis toxin (PTx) (0.34 μg in 0.5 μl). Each column represents the mean (bars show s.e.mean), of at least 6 observations. Groups of (a) vehicle- and (b–f) PTx-treated rats were previously analysed by use of ANOVA followed, where appropriate, by paired Student's *t* test. Significance of the differences of total voltage power between pretreatment period and post-drug periods are denoted: * $P < 0.05$.

lasting behavioural sedation without an increase in total voltage power and that in preselected frequency bands.

Discussion

The signal translation of α_2-adrenoceptor occupation into a specific cell response negatively influences adenylate cyclase via the inhibitory binding regulatory protein (Gi) in a variety of tissues (Jakobs *et al.*, 1976; Aktories *et al.*, 1980; Yamazaky *et al.*, 1982; Kurose *et al.*, 1983). Pertussis toxin (islet-activating protein) is well known to prevent receptor-mediated adenylate cyclase inhibition by ADP-ribosylation of a nucleotide-binding protein (Gi) in the cell membrane (Hazeki & Ui, 1981; Jakobs *et al.*, 1984). In addition, it has been demonstrated that Gi regulates the binding affinity of the agonist and antagonist to α_2-adrenoceptors in cerebral cortical membranes (Nomura *et al.*, 1985).

The present data clearly show that PTx injected into the LC of the rat significantly reduced or abolished the effects of clonidine and yohimbine, two specific compounds acting on α_2-adrenoceptors. Our study also showed that behavioural and ECoG soporific effects induced by intraperitoneal administration of clonidine were markedly decreased in rats pretreated with PTx. This strongly supports our hypothesis that the locus coeruleus plays a major

Table 1 Effects of clonidine (0.56 and 1.2 nmol) injected into the locus coeruleus from control (vehicle) and pertussis toxin (PTx)-pretreated rats

Drug pretreatment	*Time* (days)	*Clonidine* (nmol)	*% increase of baseline total voltage power 1 h after clonidine*	*2 h after clonidine*	*Number of animals*
Vehicle	1	0.56	+40.7 ± 4.1	+33.6 ± 4.5	8
Vehicle	1	1.2	+68.2 ± 6.8	+57.4 ± 5.7	8
PTx	1	0.56	+35.4 ± 3.9	+21.7 ± 3.2	8
PTx	1	1.2	+57.6 ± 6.6	+39.3 ± 4.1	8
Vehicle	2	0.56	+40.4 ± 4.2	+31.9 ± 4.0	6
PTx	2	0.56	−3.5 ± 2.2**	−1.7 ± 1.9**	6
Vehicle	3	0.56	+41.6 ± 4.6	+32.3 ± 3.7	6
PTx	3	0.56	−2.8 ± 2.4**	−2.7 ± 1.6**	6
Vehicle	6	0.56	+39.7 ± 3.9	+32.2 ± 4.6	8
Vehicle	6	1.2	+66.3 ± 7.1	+58.6 ± 6.1	6
PTx	6	0.56	−3.9 ± 2.0**	−2.3 ± 1.7**	8
PTx	6	1.2	+3.5 ± 2.7**	+4.4 ± 2.2**	6
Vehicle	10	0.56	+40.4 ± 4.3	+32.1 ± 4.3	8
Vehicle	10	1.2	+67.2 ± 6.7	+56.4 ± 6.4	8
PTx	10	0.56	+41.2 ± 3.8	+31.9 ± 4.1	8
PTx	10	1.2	+68.1 ± 6.4	+56.5 ± 6.2	8

Pertussis toxin (0.34 μg) or vehicle (phosphate buffer 67 mM) was injected into the locus coeruleus 1, 2, 3, 6 and 10 days before testing. The results are presented as mean values of the % increase of baseline total voltage power 1 h and 2 h after clondine in comparison to control period in PTx- and vehicle-treated rats. Significant differences relative to concurrent vehicle and PTx-groups are denoted: **$P < 0.01$ (unpaired Student's t test).

role in the soporific effects of clonidine (De Sarro *et al.*, 1987).

The antagonistic effects of PTx required some time to take effect, since 24 h after PTx pretreatment the ECoG changes induced by clonidine or yohimbine were still present whereas they were significantly reduced 2, 3 and 6 days after PTx pretreatment. This phenomenon may reflect the slow penetration of the toxin molecules into the cell membrane before reaching their ultimate site of action on intracellular membrane surface, and/or a lag period between the activation of PTx and the induction of its biochemical effects on Gi as previously suggested (Katada & Ui, 1982; Lujian *et al.*, 1984; Fujita *et al.*, 1985; Parenti *et al.*, 1986). Some authors have shown that in radioligand binding studies PTx reduces the affinity of α_2-adrenoceptor agonists, probably as a consequence of uncoupling Gi from the receptor (Boyer *et*

Table 2 Effects of yohimbine (1.3 and 2.6 nmol) injected into the locus coeruleus from control (vehicle) and pertussis toxin (PTx) pretreated rats

Drug pretreatment	*Time* (days)	*Yohimbine* (nmol)	*% decrease of baseline total voltage power 1 h after yohimbine*	*2 h after yohimbine*	*Number of animals*
Vehicle	1	1.3	−27.4 ± 2.4	−2.6 ± 2.0	8
Vehicle	1	2.6	−39.2 ± 3.6	−21.4 ± 2.2	8
PTx	1	1.3	−26.4 ± 2.9	−2.7 ± 2.2	8
PTx	1	2.6	−38.6 ± 3.8	−20.3 ± 2.6	8
Vehicle	2	1.3	−26.9 ± 2.5	−2.4 ± 2.3	6
PTx	2	1.3	+2.5 ± 1.3**	−2.9 ± 1.8**	6
Vehicle	3	1.3	−27.6 ± 2.3	−2.3 ± 1.9	6
PTx	3	1.3	+3.7 ± 1.5**	+2.4 ± 1.8**	6
Vehicle	6	1.3	−26.7 ± 2.4	−2.2 ± 1.7	8
PTx	6	1.3	+13.3 ± 3.4**	+9.6 ± 1.6**	6
Vehicle	10	1.3	−27.2 ± 2.6	−2.5 ± 1.7	6
PTx	10	1.3	−28.3 ± 3.7	−3.1 ± 2.1	6

Pertussis toxin (0.34 μg) or vehicle (phosphate buffer 67 mM) was injected into the locus coeruleus 1, 2, 3, 6 and 10 days before testing. The results are presented as mean values of the % increase of baseline total voltage power 1 h and 2 h after yohimbine in comparison to control period in PTx- and vehicle-treated rats. Significant differences between the concurrent vehicle and PTx-groups are denoted: **$P < 0.01$ (unpaired Student's t test).

al., 1982; Kurose et al., 1983; Garcia-Sainz et al., 1984; Nomura et al., 1985). Both the inhibition of the GTPase and/or the uncoupling of Gi from the receptor with the subsequent reduction of receptor affinity should decrease the effects of clonidine and yohimbine in PTx-treated rats. Pretreatment with PTx was also able to prevent the hyperpolarizing effects of the microiontophoretic application of clonidine on LC neurones (Aghajanian & Wang, 1986). The latter and our data suggest that the regulatory Gi protein, the multicomponent adenylate cyclase system or other biochemical events associated with G proteins, might be primarily involved in the genesis of the behavioural and ECoG spectrum power effects elicited at the level of the locus coeruleus by drugs acting on α_2-adrenoceptors.

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References

ABOOD, M.E., LAW, P.Y. & LOH, H.H. (1985). Pertussis toxin treatment modified opiate action in the rat brain striatum. *Biochem. Biophys. Res. Commun.*, **127**, 477–483.

AGHAJANIAN, G.K. & WANG, Y.Y. (1986). Pertussis toxin blocks the outward currents evoked by opiate and α_2-agonists in locus coeruleus neurons. *Brain Res.*, **371**, 390–394.

AKTORIES, K., SCHULTZ, G. & JAKOBS, K.H. (1980). Regulation of adenylate cyclase activity in hamster adipocytes: inhibition by prostaglandins, α-adrenergic agonists and nicotinic acid. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **312**, 167–173.

BOKOCH, G.M., KATADA, T., NORTHUP, J.K., HEWLET, E.L. & GILMAN, A.G. (1983). Identification of the predominant substrate for ADP-ribosylation by islet activating protein. *J. Biol. Chem.*, **258**, 2072.

BOYER, J.L., POSADA, C. & GARCIA-SAINZ, J.A. (1982). Effect of pertussis vaccine on α_2-adrenoceptors of the circulatory system of the rat. *Eur. J. Pharmacol.*, **83**, 123–126.

DE SARRO, G.B., ASCIOTI, C., FROIO, F., LIBRI, V. & NISTICÒ, G. (1987). Evidence that locus coeruleus is the site where clonidine and drugs acting at α_1- and α_2-adrenoceptors affect sleep and arousal mechanisms. *Br. J. Pharmacol.*, **90**, 675–685.

FUJITA, N., NAKAHIRO, M., FURUCHI, I., SAITO, K. & YOSHIDA, H. (1985). Effects of pertussis toxin on D2-dopamine receptor in rat striatum: evidence for coupling of Ni regulatory protein with D2-receptor. *Brain Res.*, **333**, 231–236.

GARCIA-SAINZ, J.A., BOYER, J.L., MICHEL, T., SAWYER, D., STILES, G.L., DOHLMANN, H. & LEFKOWITZ, R.J. (1984). Effect of pertussis toxin on α_2-adrenoceptors: decreased formation of the high-affinity state for agonists. *FEBS Lett.*, **172**, 95–98.

HAZEKI, O. & UI, M. (1981). Modification by islet-activating protein of receptor-mediated regulation of cyclic AMP accumulation in isolated rat heart cells. *J. Biol. Chem.*, **256**, 2856–2862.

JAKOBS, K.H., AKTORIES, K. & SCHULTZ, G. (1984). Mechanism of pertussis toxin action on the adenylate cyclase system. *Eur. J. Biochem.*, **140**, 177–181.

JAKOBS, K.H., SAUR, W. & SCHULTZ, G. (1976). Reduction of adenylate cyclase activity in lysates of human platelet by the α-adrenergic component of epinephrine. *J. Cyclic Nucleotide Res.*, **2**, 381–392.

KATADA, T. & UI, M. (1982). Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 3127–3133.

KUROSE, H., KATADA, T., AMANO, T. & UI, M. (1983). Specific uncoupling by islet-activation protein, pertussis toxin, of negative signal transduction via α-adrenergic, cholinergic and opiate receptors. *J. Biol. Chem.*, **258**, 4870–4875.

LUJIAN, M., LOPEZ, E., RAMIREZ, R., ANGUILAR, H., MARTINEZ-OLMEDO, M.D. & GARCA-SAINZ, J.A. (1984). Pertussis toxin blocks the action of morphine, norepinephrine and clonidine on isolated guinea-pig ileum. *Eur. J. Pharmacol.*, **100**, 377–380.

NOMURA, Y., KITAMURA, Y. & SEGAWA, T. (1985). Decrease of clonidine binding affinity to α-adrenoceptor by ADP-ribosylation of 41.000 dalton proteins in rat cerebral cortical membranes by islet-activating protein. *J. Neurochem.*, **44**, 354–369.

OHTA, H., OKAJIMA, F. & UI, M. (1985). Inhibition by islet activating protein of a chemotactic peptide-induced early breakdown of inositol phospholipids and Ca^{++} mobilization in guinea-pig neutrophils. *J. Biol. Chem.*, **260**, 15771–15780.

PARENTI, M., TIRONE, F., GIAGNONI, G., PECORA, N. & PAROLARO, D. (1986). Pertussis toxin inhibits the antinociceptive action of morphine in the rat. *Eur. J. Pharmacol.*, **124**, 357–359.

PAXINOS, F. & WATSON, C. (1982). *The Rat Brain in Stereotaxic Coordinates.* London: Academic Press.

TALLARIDA, R.J. & MURRAY, R.B. (1981). *Manual of Pharmacological Calculations with Computer Programs.* Berlin: Springer-Verlag.

YAMAZAKY, S., KATADA, T. & UI, M. (1982). Alpha (2)-adrenergic inhibition of insulin secretion via interference with cyclic AMP generation in rat pancreatic islets. *Mol. Pharmacol.*, **21**, 618–653.

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